

Methodology article

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## PCR and real-time PCR primers developed for detection and identification of *Bifidobacterium thermophilum* in faeces

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### Abstract

**Background:** Culture-independent methods based on the 16S ribosomal RNA molecule are nowadays widely used for assessment of the composition of the intestinal microbiota, in relation to host health or probiotic efficacy. Because *Bifidobacterium thermophilum* was only recently isolated from human faeces until now, no specific real-time PCR (qPCR) assay has been developed for detection of this species as component of the bifidobacterial community of the human intestinal flora.

**Results:** Design of specific primers and probe was achieved based on comparison of 108 published bifidobacterial 16S rDNA sequences with the recently published sequence of the human faecal isolate *B. thermophilum* RBL67. Specificity of the primer was tested *in silico* by similarity search against the sequence database and confirmed experimentally by PCR amplification on 17 *Bifidobacterium* strains, representing 12 different species, and two *Lactobacillus* strains. The qPCR assay developed was linear for *B. thermophilum* RBL67 DNA quantities ranging from 0.02 ng/μl to 200 ng/μl and showed a detection limit of 10<sup>5</sup> cells per gram faeces. The application of this new qPCR assay allowed to detect the presence of *B. thermophilum* in one sample from a 6-month old breast-fed baby among 17 human faecal samples tested. Additionally, the specific qPCR primers in combination with selective plating experiments led to the isolation of F9K9, a faecal isolate from a 4-month old breast-fed baby. The 16S rDNA sequence of this isolate is 99.93% similar to that of *B. thermophilum* RBL67 and confirmed the applicability of the new qPCR assay in faecal samples.

**Conclusion:** A new *B. thermophilum*-specific qPCR assay was developed based on species-specific target nucleotides in the 16S rDNA. It can be used to further characterize the composition of the bifidobacterial community in the human gastrointestinal tract. Until recently, *B. thermophilum* was considered as a species of animal origin, but here we confirm with the application of this new PCR assay the presence of *B. thermophilum* strains in the human gut.

### Background

Real-time quantitative polymerase chain reaction (qPCR) has recently emerged as promising tool for faecal microbiota monitoring in animal and human faeces [1-3] since

culture-based methods are not suitable for quantification of certain microbial groups, species or strains in faeces [4]. Due to the role of bifidobacteria as probiotics much attention has been focused on the qPCR-based quantification

of both the autochthonous bifidobacteria in faecal microbiota and on selected strains of bifidobacteria after consumption as probiotics [5-9]. Compared to fluorescence *in situ* hybridization (FISH), the most widely used method for culture independent quantification in faeces, qPCR is less developed in terms of the availability of specific probes [10]. On the other hand qPCR was shown to be about a 10 to 100 fold more sensitive than culture- and FISH-based enumeration techniques [11], as well as to be rapid, easy and more accurate for quantification of low levels of bacteria [12]. Several oligonucleotides were designed for the *Bifidobacterium* species found in the human intestinal tract, most of them based on the 16S rDNA sequence [11,13]. Other target sequences like the transaldolase encoding gene [5], heat-shock protein (HSP60) gene [14], intergenic spacer of the 16S-23S rRNA gene [15] are also being investigated for species-specific detection and quantification. Oligonucleotides targeting such sequences could also be used for developing qPCR primers.

*Bifidobacterium thermophilum*, being considered as an animal-associated commensal species, was never included in studies on the bifidobacterial composition of the human intestinal flora and to our knowledge, no oligonucleotide was designed for the development of *B. thermophilum*-specific PCR or qPCR assay until now. Recently, design of a pair of oligonucleotides for PCR amplification of a portion of the 16S rDNA of *B. thermophilum* was reported, but effective specificity of the assay was questioned [16]. Previously, we have isolated and characterized bifidobacteria with anti-*Listeria* activity from stool of newborns [17,18]. Strain RBL67 was identified as *B. thermophilum* using 16S rDNA sequence homology, comparative HSP60 sequence analysis, DNA-DNA genome hybridization and carbohydrate fermentation patterns [19]. This was the first demonstration of the presence of *B. thermophilum* in human faeces. In this study, we designed oligonucleotides specific

for *B. thermophilum* that we used to develop PCR and qPCR assays to study the distribution of this species in human faecal samples. Finally, the qPCR technology let us isolate a strain of *B. thermophilum* from human infant faeces closely related to *B. thermophilum* RBL67.

## Results

### Design of a *B. thermophilum* specific PCR assay

Specificity of the *B. thermophilum* specific primer was assessed by PCR on colonies with primers btherm and the *Bifidobacterium* genus-specific primer lm3 (Table 1). Of the 17 *Bifidobacterium* and two *Lactobacillus* strains tested, positive signals (amplification of a fragment of approximately 1.5 kb) were only obtained with the three faecal isolates *B. thermophilum* RBL67, *Bifidobacterium thermacidophilum* subsp. *porcinum* RBL68 and RBL70 (Figure 2, lanes 1 to 3, respectively), *B. thermophilum* DSM20210<sup>T</sup> (Figure 2, lane 6), and *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup> (Figure 2, lane 5).

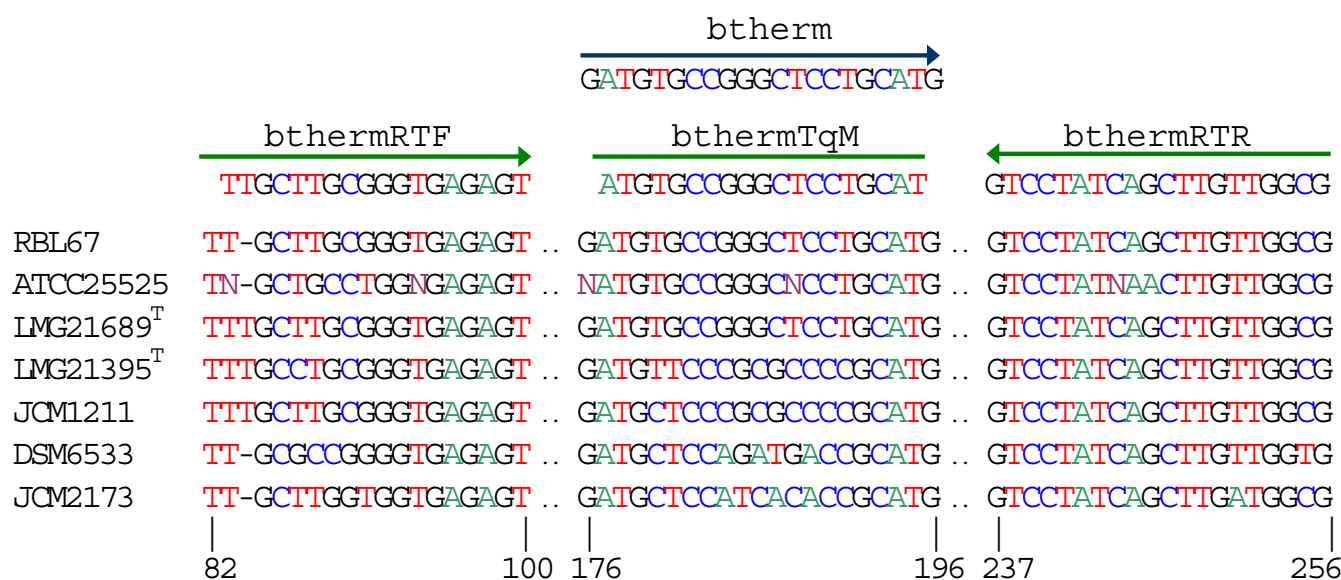
### Detection of *B. thermophilum* in faecal DNA samples by PCR

Classical PCR analysis with the *B. thermophilum* specific primers btherm and lm3 (Table 1) on total DNA isolated from faecal samples spiked with known quantities of *B. thermophilum* RBL67 showed that the detection limit of the method was 10<sup>8</sup> *B. thermophilum* cells per gram faeces (Figure 3). This high detection limit did not allow DNA amplification from any of the 17 faecal samples. Efficacy of PCR amplification on faecal DNA samples was confirmed by amplification of a 1.3 kb-DNA fragment from each faecal DNA sample generated with the *Bifidobacterium*-genus specific primer-pair lm26/lm3 [20] (data not shown).

**Table 1: Oligonucleotides used in this study**

Oligonucleotide	Sequence 5'-3'	Reference
btherm	GAT GTG CCG GGC TCC TGC ATG	This study
lm3	CGG GTG CTI CCC ACT TTC ATG	[20]
lm26	GAT TCT GGC TCA GGA TGA ACG	[20]
bak1lw	AGT TTG ATC MTG GCT CAG	[21]
bak4	AGG AGG TGA TCC ARC CGC A	[22]
bthermRTF	TTG CTT GCG GGT GAG AGT	This study
bthermRTR	CGC CAA CAA GCT GAT AGG AC	This study
bthermTqM	*FAM-ATG TGC CGG GCT CCT GCA T-*TAMRA	This study
520F	CAG GAG TGC CAG CAG CCG CGG	[23]
520R	ACC GCG GCT GCT GGC	[23]
1100F	CAG GAG CAA CGA GCG CAA CCC	[23]
1100R	AGG GTT GCG CTC GTT G	[23]

\*FAM (6-carboxyfluorescein): fluorescent reporter dye. TAMRA (6-carboxytetramethylrhodamine): quencher.

**Figure 1**

**Localization of the 16S rDNA targets for oligonucleotides designed in this study.** Multiple alignment of 16S rDNA sequences of *B. thermophilum* RBL67 [GenBank: [DQ340557.1](#)], *B. thermophilum* ATCC25525<sup>T</sup> [GenBank: [U10151.1](#)], *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup> [GenBank: [AY148470.1](#)], *B. thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup> [GenBank: [AB016246.1](#)], *B. boum* JCM1211 [GenBank: [D86190.1](#)], *Bifidobacterium saeculare* DSM6533 [GenBank: [D89330.1](#)] and *B. breve* JCM1273 [GenBank: [AF491832](#)]. Numbers correspond to *E. coli* 16S rDNA positions.

#### Development of a qPCR assay for detection of *B. thermophilum* in human faeces

The qPCR technology was chosen as an alternative to the classical PCR for its higher sensitivity. *B. thermophilum* specificity of qPCR performed with primers bthermRTF, bthermRTR and the TaqMan probe bthermTqM (Table 1) was tested by amplification of DNA isolated from six different *Bifidobacterium* strains belonging to four closely related species. A positive signal was obtained for *B. thermophilum* RBL67 ( $C_T = 17.3 \pm 0.5$ ), *B. thermophilum* DSM20210<sup>T</sup> ( $C_T = 24.9 \pm 0.3$ ) and the closely related species *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup> ( $C_T = 16.3 \pm 0.4$ ), but not for *B. thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup>, *Bifidobacterium breve* DSM20213<sup>T</sup>, and *Bifidobacterium boum* DSM20432<sup>T</sup>. Amplification of DNA from *B. thermophilum* RBL67 with this new assay was shown to be linear for DNA concentrations ranging from 0.02 ng/ $\mu$ l ( $C_T = 28.1 \pm 0.3$ ) to 200 ng/ $\mu$ l ( $C_T = 15.3 \pm 0.4$ ) with a regression coefficient  $R^2 = 0.991$  (data not shown).

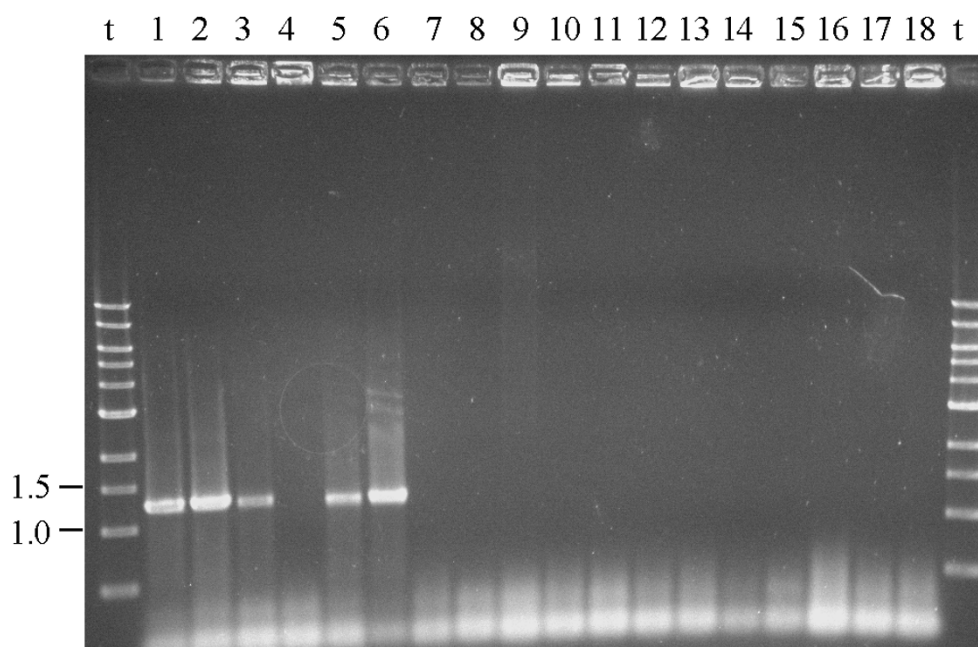
#### Screening of faecal samples by qPCR

As for the classical PCR approach, the detection limit of the qPCR assay was determined by analysis of DNA isolated from spiked faecal samples and was equal to  $1 \times 10^5$  bacterial cells per gram of faeces. Detection in faecal samples was shown to be linear between  $1 \times 10^9$  and  $1 \times 10^5$

cells per gram of faeces and corresponded to  $C_T$  values comprised between  $21.6 \pm 0.6$  and  $33.8 \pm 0.1$ , respectively, with a regression coefficient  $R^2 = 0.995$  (data not shown). One of the 17 faecal samples (sample C7 from a 6 month-old breast-fed baby) gave a positive signal within this range (Figure 4) with a  $C_T$  value of  $28.6 \pm 0.3$ , corresponding to a concentration of  $5 \times 10^6$  cells per gram faeces.

#### Application of the newly developed qPCR assay for faecal samples

Raffinose-*Bifidobacterium* (RB) medium and MRSC-NNLP were tested for isolation of new *B. thermophilum* strains from human faeces. MRSC-NNLP was chosen for isolation of bifidobacteria because it allowed a better growth of RBL67 ( $1.5 \times 10^9$  cfu/ml after three days of incubation at 40°C, in comparison to  $2.9 \times 10^7$  cfu/ml in RB-medium and under the same conditions) and was easier to prepare. Approximately  $1-5 \times 10^8$  colonies per gram faeces could be cultivated from all of the 17 samples plated on MRSC-NNLP agar. Microscopic observations of the isolates showed that the medium was not completely selective, allowing for the growth of non-rod-shaped microorganisms. 60 rod-shaped microorganisms were selected for PCR analysis on colony. Twenty-five of them were positive with the *Bifidobacterium* genus specific primers Im26/Im3, but only one of them gave a positive signal with

**Figure 2**

**Specificity of the PCR for *B. thermophilum* using primers btherm and lm3.** Agarose gel electrophoresis of PCR products (1.3 kb) obtained with primers btherm/lm3 on colonies of *B. thermophilum* RBL67 (1), *B. thermacidophilum* subsp. *porcinum* RBL68 (2), *B. thermacidophilum* subsp. *porcinum* RBL70 (3), *B. thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup> (4), *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup> (5), *B. thermophilum* DSM20210<sup>T</sup> (6), *B. boum* DSM20432<sup>T</sup> (7), *B. breve* DSM20213<sup>T</sup> (8), *Bifidobacterium longum* NCC2705 (9), *Bifidobacterium coryneforme* DSM2026<sup>T</sup> (10), *Bifidobacterium asteroides* DSM20089<sup>T</sup> (11), *Bifidobacterium animalis* subsp. *lactis* DSM10140 (12), *B. animalis* subsp. *animalis* DSM20105 (13), *Bifidobacterium cuniculi* DSM20435<sup>T</sup> (14), *Bifidobacterium adolescentis* DSM20083<sup>T</sup> (15), *Bifidobacterium bifidum* DSM20456<sup>T</sup> (16), *Lactobacillus delbrueckii* subsp. *lactis* DSM20072<sup>T</sup> (17) and *Lactobacillus plantarum* subsp. *plantarum* DSM20174<sup>T</sup> (18), t: Tridye 1-kb DNA ladder, in kb (New England Biolabs, Ipswich, MA, USA).

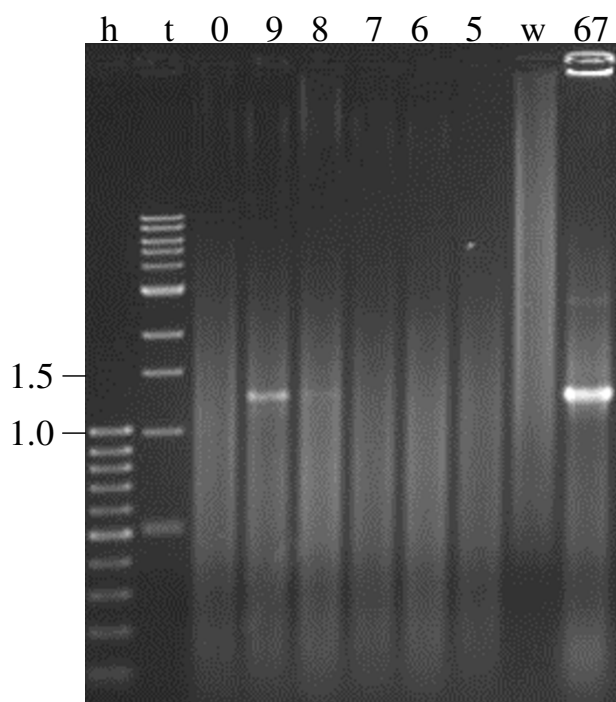
btherm/lm3. This isolate, F9K9 from a 4-month old breast-fed baby, was streaked several times on MRSC agar and the absence of contaminants other than *Bifidobacterium* was confirmed by three PCR reactions with lm26/lm3, btherm/lm3 and bak4/bak11w [24] (data not shown). Sequencing of the 16S rDNA fragment amplified with lm26 and lm3 yielded a 1454-bp sequence which was 99.93% identical to the 16S rDNA of *B. thermophilum* RBL67. Sequence identities with other *Bifidobacterium* strains are summarized in Table 2.

## Discussion

Real-time PCR (qPCR) is known to be a more sensitive technique than classical PCR. This is reflected by our results for specific amplification of 16S rDNA from spiked faecal samples, where changing from classical PCR to qPCR for the detection of *B. thermophilum* in faecal samples lowered the detection limit of the assay from  $10^8$  to  $10^5$  cells per gram faeces. The high sensitivity obtained for qPCR in this study is similar to detection limits reported by different groups for other *Bifidobacterium* species or

genus specific qPCR assays. Matsuki *et al.* [11], Penders *et al.* [6] and Gueimonde *et al.* [25], for example, reported detection limits of  $10^6$ ,  $5 \times 10^3$  and  $5 \times 10^4$  cells of *Bifidobacterium* spp. per gram faeces, respectively. The application of a recent qPCR technology using rRNA as target molecule combined with reverse transcriptase could further enhance the sensitivity down to  $10^3$  cells/g faeces [26]. This methodology could also be developed for the detection of other subdominant faecal bacteria such as *B. thermophilum*.

We have developed a qPCR assay which is specific for *B. thermophilum* although the assay is also positive for the type strain of *B. thermacidophilum* subsp. *porcinum*, and *B. thermacidophilum* subsp. *porcinum* RBL68 and RBL70 (these subspecies were originally named "suis" which is a synonym for "porcinum"), but not with *B. thermacidophilum* subsp. *thermacidophilum* (LMG21395<sup>T</sup>) [27]. Based on our published data including DNA-DNA genome hybridizations [19] we underline that *B. thermacidophilum* subsp. *porcinum* [28] should belong to the *B. thermophilum* spe-



**Figure 3**  
**Determination of the detection limit of the *B. thermophilum* PCR on faecal samples.** PCR amplification with primers btherm and lm3 of DNA isolated from faecal sample F0, the PCR-amplification negative control (0) spiked with  $10^9$  (9),  $10^8$  (8),  $10^7$  (7),  $10^6$  (6) or  $10^5$  (5) *B. thermophilum* RBL67 cells/g faeces; h: 100-bp DNA ladder [kb]; t: 1-kb DNA ladder [kb]; w: water; 67: PCR on a colony of *B. thermophilum* RBL67.

cies and consequently, we conclude that our qPCR system is specific for *B. thermophilum*.

Until now, *B. thermophilum* was considered as an animal-associated species, mainly present in faeces of ruminants and pigs. The amplification of a specific 16S rDNA sequence with our qPCR on the children faecal sample C7 as well as the isolation of a *B. thermophilum* isolate from children faeces during this work support the assumption of von Ah *et al.* [19] that presence of *B. thermophilum* in food cannot be used to discriminate between animal and human bacterial contamination, as previously suggested [29].

## Conclusion

This is the first report of the development of a qPCR assay for specific detection of *B. thermophilum*, a species that was not included in analysis of the composition of the bifidobacterial human intestinal microbiota until now. Using this assay, we detected *B. thermophilum* at a concentration

of  $5 \times 10^6$  cells per gram in one faeces sample, confirming the presence of this species in human faecal material.

## Methods

### Bacterial strains and culture conditions

Unless otherwise indicated, bifidobacteria and lactobacilli were grown in liquid cultures overnight in 10 ml MRSC medium consisting of MRS [30], obtained from Biolife (Milan, Italy) and supplemented with 0.05% L-cysteine hydrochloride, or on MRSC-agar plates (MRSC supplemented with 1.5% w/v agar). Incubation was carried out for 24 h at 37°C in anaerobic jars with an anaerobic atmosphere generation system (Oxoid AnaeroGen TM, Basel, Switzerland). *B. thermophilum* RBL67, as well as *B. thermophilum* subsp. *porcinum* RBL68 and RBL70 are human infant faecal isolates [17-19,31]. All of the other strains are commercial strains from DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany) or LMG (Laboratories for Microbiology and Microbial Genetics, Ghent, Belgium).

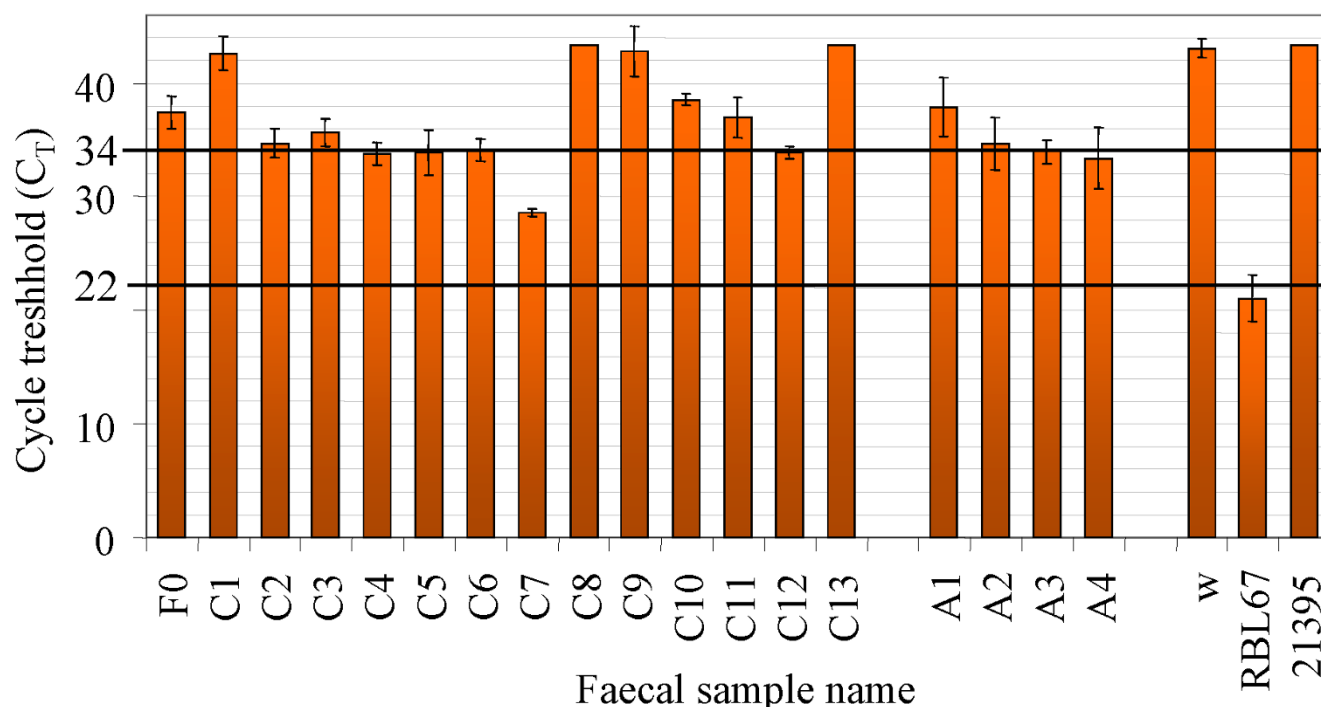
### Isolation of bifidobacteria from faecal samples

Seventeen faecal samples from human adults (4) and breast-fed children between 1 to 6 months (13) were collected as already described [32]. Subjects or parents of the subjects were informed orally and in writing about the aims and procedures of the study and consent was obtained from them. The study protocol was reviewed and approved by the Ethical Committees of the canton of Zurich and the SPUK-committee of the University Children's Hospital of Zurich (project StV31/05).

For efficient growth of *B. thermophilum* strains from faecal samples, Raffinose-Bifidobacterium (RB) [33] and MRSC-NNLP [34] media were compared. Serial 10-fold dilutions of overnight cultures of *B. thermophilum* RBL67 (containing approximately  $10^9$  cfu/ml) in saline solution (8.5 g/Liter NaCl, 1 g/Liter peptone, 0.05% cysteine-HCl, pH 6–7) were plated on RB and on MRSC-NNLP, incubated for 3 days anaerobically at 40°C and cell counts were determined. Incubation temperature of 40°C was chosen as an additional selective condition, due to the relative heat tolerance of *B. thermophilum* spp. [19]. For isolation of bifidobacteria from faecal samples, 20 mg of samples were homogenized by vigorous vortexing in 0.2 ml of saline solution, 10-fold serially diluted in the same solution and spread on MRSC-NNLP agar plates. Plates were incubated for 3 days under anaerobic conditions at 40°C and single isolates were observed under light microscope. Rod-shaped bacteria were selected for further analysis.

### DNA purification methods

Total DNA was isolated from pure cultures of *B. thermophilum* RBL67, *B. thermophilum* DSM20210<sup>T</sup>, *Bifidobacterium thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup>, *B.*

**Figure 4**

**Detection of *B. thermophilum* in faecal samples by qPCR targeting the 16S rDNA.** Cycle threshold ( $C_T$ ) values measured for faecal samples from 13 children (C1 to C13) and 4 adults (A1 to A4). F0: PCR-negative faecal sample, w: water instead of DNA, RBL67: DNA from pure culture of RBL67, 21395: DNA from *B. thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup>. Values are means and standard deviations for three repetitions of the qPCR assay with three replicates each.

*thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup>, *Bifidobacterium breve* DSM20213<sup>T</sup> and *Bifidobacterium boum* DSM20432<sup>T</sup> according to Leenhouts *et al.* [35]. Total DNA was prepared from 200 mg of 17 faecal samples using the QiAamp DNA Stool Mini kit (Qiagen, Basel, Switzerland) according to manufacturer's instructions. A PCR-amplification negative faecal sample (F0) was pre-

pared by autoclaving twice one of the samples. For determination of the detection limit, 10- $\mu$ l aliquots of F0 were spiked before DNA preparation with a 10-fold serial dilution of *B. thermophilum* RBL67 (overnight culture in MRSC) at concentrations ranging from 10<sup>9</sup> to 10<sup>1</sup> bacterial cells per g faeces. The extracted DNA was stored at -20°C.

**Table 2: 16S rDNA sequence identities of isolate F9K9 with published 16S rDNA sequences**

Accession	Description	% I*
<a href="#">DQ340557.1</a>	<i>B. thermophilum</i> RBL67 [19]	99.93
<a href="#">AY148470.1</a>	<i>B. thermacidophilum</i> subsp. <i>porcinum</i> LMG21689 <sup>T</sup> [28]	99.64
<a href="#">D86190.1</a>	<i>B. boum</i> JCM1211 (DSM20432 <sup>T</sup> )	98.15
<a href="#">AB016246.1</a>	<i>B. thermacidophilum</i> subsp. <i>thermacidophilum</i> LMG21395 <sup>T</sup> [27]	97.02
<a href="#">U10151.1</a>	<i>B. thermophilum</i> ATCC25525 <sup>T</sup> (DSM20210 <sup>T</sup> )	95.82
<a href="#">D89330.1</a>	<i>B. saeculare</i> DSM6533	95.29
<a href="#">AF491832</a>	<i>B. breve</i> JCM1273 (= DSM20091)	95.16

\*The percentage of identity (% I) was determined by comparison of the sequence of F9K9 against the sequences present in the database with the BLAST tool from NCBI.

### DNA sequencing, PCR and qPCR reactions

Primers and probe used in this study were synthesized by Microsynth and are listed in Table 1. The TaqMan probe bthermTqM was labeled with 5'-FAM as fluorescent reporter dye and 3'-TAMRA as quencher. Classical PCR was performed either on 2 µl DNA prepared from faecal samples as described above, or on 40 µl cell suspensions. For that, one colony was picked from an agar plate and resuspended in 210 µl of sterile, double distilled water. A 50-µl classical PCR reaction consisted of 2.5 U EuroTaq-DNA-Polymerase (Digitana, Horgen, Switzerland), 1.5 mM magnesium chloride (Digitana), 0.2 mM dNTP's (GE Healthcare) and 0.5 µM of each primer. When DNA isolated from faecal samples was used as template, 0.1 µg/ml BSA was added to the PCR reaction. Amplification conditions were as follows: 3 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 62°C and 2 min at 72°C, followed by 7 min at 72°C. Sequencing of the PCR product for 16S rDNA was performed by Microsynth (Balgach, Switzerland) using the primers btherm, 520F, 520R, 1100F, 1100R and Im3 (Table 1).

The qPCR reactions were set in a total volume of 25 µl, containing 2.5 µl of DNA extracted from faecal samples with the stool kit as described above, 12.5 µl of qPCR MasterMix from Eurogentec (Seraing, Belgium), 0.3 µM of each primer and 0.1 µM of the TaqMan probe. Reactions were run on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland). The amplification conditions were 2 min at 50°C, 10 min denaturation at 95°C, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. The cycle threshold ( $C_T$ ), corresponding to the number of cycles after which the target-DNA concentration increase becomes exponential, was monitored. Results were analyzed using the SDS 2.1 Software (Applied Biosystems). All reactions were done in triplicate and repeated at least twice (three times when faecal DNA extract was used as a template). Values in the text are mean  $\pm$  SD.

### Design of a *B. thermophilum* specific primer and qPCR assay

One hundred and eight sequences of bifidobacterial 16S rDNA of more than 1 kb in length were retrieved from the EMBL nucleotide sequence database <http://www.ebi.ac.uk/embl> and were used, together with the sequence of the 16S rDNA of *B. thermophilum* RBL67 [GenBank: DQ340557], to prepare a multiple alignment with ClustalW in the sequence analysis program BioEdit [36]. The primer btherm (Table 1) was manually designed in a variable region. Specificity of the primer was verified *in silico* with FASTA and the BLAST program from NCBI (version 2.2.15). For *B. thermophilum*-specific PCR amplification, primer btherm was used together with the *Bifidobacterium*-genus-specific reverse primer Im3. Cell

suspensions of *B. thermophilum* RBL67 and DSM20210<sup>T</sup>, *B. thermacidophilum* strains RBL68, RBL70, LMG21395<sup>T</sup> and LMG21689<sup>T</sup>, *B. boum* DSM20432<sup>T</sup>, *B. breve* DSM20213<sup>T</sup>, *B. longum* DSM20219<sup>T</sup>, *B. coryneforme* DSM2026<sup>T</sup>, *B. asteroides* DSMZ20089<sup>T</sup>, *B. animalis* subsp. *lactis* DSM10140, *B. animalis* subsp. *animalis* DSM20105, *B. cuniculi* DSM20435<sup>T</sup>, *B. adolescentis* DSM20083<sup>T</sup>, *B. bifidum* DSM20456<sup>T</sup>, *Lactobacillus delbrueckii* subsp. *lactis* DSM20072<sup>T</sup> and *Lactobacillus plantarum* subsp. *plantarum* DSM20174<sup>T</sup> were used as template for amplification with btherm and Im3 under the conditions described below to test the specificity of the PCR. For the development of the qPCR assay, the btherm primer was modified to fit the lower melting temperature required for a TaqMan probe and adequate adjacent forward and reverse primers were designed with the program Primer3 [37] (Table 1). Aliquots of 5 µl of DNA (20 ng/µl) isolated from pure cultures of *B. thermophilum* RBL67, *B. thermophilum* DSM20210<sup>T</sup>, *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup>, *B. thermacidophilum* subsp. *thermacidophilum* LMG21395<sup>T</sup>, *B. breve* DSM20213<sup>T</sup> and *B. boum* DSM20432<sup>T</sup> were amplified with this assay to assess its specificity. Localization of the primers btherm, bthermRTF and bthermRTR and of the TaqMan probe bthermTqM on an alignment of 16S rDNA sequences of seven bifidobacteria is shown in Figure 1.

### Authors' contributions

SM participated in the study conception and coordination, drafted the manuscript and designed the specific oligonucleotides. RM performed part of the experiments. LM and CL provided guidance during all parts of the work. All authors read and approved the final manuscript.

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### References

1. Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K, Tanaka R: **Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria.** *Appl Environ Microbiol* 2004, **70**:167-173.
2. Hopkins MJ, Macfarlane GT, Furrer E, Fite A, Macfarlane S: **Characterisation of intestinal bacteria in infant stools using real-time PCR and Northern hybridisation analyses.** *FEMS Microbiol Ecol* 2005, **54**:77-85.
3. Kawaji S, Taylor DL, Mori Y, Whittington RJ: **Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture.** *Vet Microbiol* 2007, **125**:36-48.
4. Blaut M, Collins MD, Welling GW, Doré J, van Loo J, de Vos W: **Molecular biological methods for studying the gut microbiota: the EU human gut flora project.** *Br J Nutr* 2002, **87**:S203-S211.
5. Requena T, Burton J, Matsuki T, Munro K, Simon MA, Tanaka R, Watanabe K, Tannock GW: **Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene.** *Appl Environ Microbiol* 2002, **68**:2420-2427.



6. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE: **Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR.** *FEMS Microbiol Lett* 2005, **243**:141-147.
7. Maruo T, Sakamoto M, Toda T, Benno Y: **Monitoring the cell number of *Lactococcus lactis* subsp. *cremoris* FC in human faeces by real-time PCR with strain-specific primers designed using the RAPD technique.** *Int J Food Microbiol* 2006, **110**:69-76.
8. Fujiimoto J, Matsuki T, Sasamoto M, Tomii Y, Watanabe K: **Identification and quantification of *Lactobacillus casei* strain Shirota in human feces with strain-specific primers derived from randomly amplified polymorphic DNA.** *Int J Food Microbiol* 2008, **126**(1-2):210-215.
9. Delroisse JM, Boulvin AL, Permentier I, Dauphin RD, et al.: **Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. in rat fecal samples by real-time PCR.** *Microbiol Res* in press.
10. Vaughan EE, Heilig HG, Ben-Amor K, de Vos WM: **Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches.** *FEMS Microbiol Rev* 2005, **29**:477-490.
11. Matsuki T, Watanabe K, Tanaka R: **Genus- and species-specific PCR primers for the detection and identification of bifidobacteria.** *Curr Issues Intest Microbiol* 2003, **4**(2):61-69.
12. Gueimonde M, Debor L, Tolkko S, Jokisalo E, Salminen S: **Quantitative assessment of faecal bifidobacterial populations by real-time PCR using lanthanide probes.** *J Appl Microbiol* 2007, **102**:1116-1122.
13. Satokari RM, Vaughan EE, Smidt H, Saarela M, Matto J, de Vos WM: **Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract.** *Syst Appl Microbiol* 2003, **26**:572-584.
14. Delcenserie V, Bechoux N, China B, Daube G, Gavini F: **A PCR method for detection of bifidobacteria in raw milk and raw milk cheese: comparison with culture-based methods.** *J Microbiol Methods* 2005, **61**:55-67.
15. Haarman M, Knol J: **Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula.** *Appl Environ Microbiol* 2005, **71**:2318-2324.
16. Youn SY, Seo JM, Ji GE: **Evaluation of the PCR method for identification of *Bifidobacterium* species.** *Lett Appl Microbiol* 2008, **46**:7-13.
17. Touré R, Kheadr E, Lacroix C, Moroni O, Fliss I: **Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*.** *J Appl Microbiol* 2003, **95**:1058-1069.
18. Kheadr E, Dabour N, von Ah U, Lacroix C, Meile L, Fliss I: **Genetic and phenotypic diversity of *Bifidobacterium thermacidophilum* fecal isolates from newborns.** *Can J Microbiol* 2007, **53**:1348-1359.
19. von Ah U, Mozzetti V, Lacroix C, Kheadr EE, Fliss I, Meile L: **Classification of a moderately oxygen-tolerant isolate from baby faeces as *Bifidobacterium thermophilum*.** *BMC Microbiol* 2007, **7**:79.
20. Kaufmann P, Pfefferkorn A, Teuber M, Meile L: **Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR.** *Appl Environ Microbiol* 1997, **63**:1268-1273.
21. Goldenberger D, Perschil I, Ritzler M, Altwegg M: **A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification.** *PCR Methods Appl* 1995, **4**:368-370.
22. Greisen K, Loeffelholz M, Purohit A, Leong D: **PCR primers and probes for the 16S rDNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid.** *J Clin Microbiol* 1994, **32**:335-351.
23. Miyake T, Watanabe K, Watanabe T, Oyaizu H: **Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences.** *Microbiol Immunol* 1998, **42**:661-667.
24. Schürch C: **Development of a novel DNA transformation system for bifidobacteria.** In *PhD thesis Nr. 14676 Swiss Federal Institute of Technology, Dept of Agricultural and Food Sciences*; 2002.
25. Gueimonde M, Tolkko S, Korpimäki T, Salminen S: **New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples.** *Appl Environ Microbiol* 2004, **70**:4165-4169.
26. Matsuda K, Tsuji H, Takashi A, Kado Y, Nomoto K: **Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR.** *Appl Environ Microbiol* 2007, **73**:32-39.
27. Dong X, Xin Y, Jian W, Liu X, Ling D: ***Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester.** *Int J Syst Evol Microbiol* 2000, **50**:119-125.
28. Zhu L, Li W, Dong X: **Species identification of genus *Bifidobacterium* based on partial HSP60 gene sequences and proposal of *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov.** *Int J Syst Evol Microbiol* 2003, **53**:1619-1623.
29. Delcenserie V, Bechoux N, Leonard T, China B, Daube G: **Discrimination between *Bifidobacterium* species from human and animal origin by PCR-restriction fragment length polymorphism.** *J Food Prot* 2004, **67**:1284-1288.
30. de Man JD, Rogosa M, Sharpe ME: **A medium for the cultivation of lactobacilli.** *J Appl Bacteriol* 1960, **23**:130-135.
31. Moroni O, Kheadr E, Boutin Y, Lacroix C, Fliss I: **Inactivation of adhesion and invasion of food-borne *Listeria monocytogenes* by bacteriocin-producing *Bifidobacterium* strains of human origin.** *Appl Environ Microbiol* 2006, **72**:6894-6901.
32. Mathys S, von Ah U, Lacroix C, Staub E, Mini R, Cereghetti T, Meile L: **Detection of the pediocin gene *pedA* in strains from human faeces by real-time PCR and characterization of *Pediococcus acidilactici* UVA1.** *BMC Biotechnol* 2007, **7**:55.
33. Hartemink R, Kok BJ, Weenk GH, Rombouts FM: **Raffinose-*Bifidobacterium* (RB) agar, a new selective medium for bifidobacteria.** *J Microbiol Methods* 1996, **27**:33-43.
34. Dave RI, Shah NP: **Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria.** *J Dairy Sci* 1996, **79**:1529-1536.
35. Leenhouts KJ, Kok J, Venema G: **Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*.** *Appl Environ Microbiol* 1989, **55**:394-400.
36. Hall TA: **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.** *Nucl Acids Symp Ser* 1999, **41**:95-98.
37. Rozen S, Skaletsky HJ: **Primer3 on the WWW for general users and for biologist programmers.** In *Bioinformatics Methods and Protocols: Methods in Molecular Biology* Edited by: Krawetz S, Misener S. Press H. Totowa; 2000:365-386.

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